

## ASSESSING PROGRESSION OF CERVICAL PRE-CANCER LESIONS

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**Abstract.** The purpose of this study was to accomplish a comparative assessment between the immune histochemical and the immunocytochemical expression of p16 protein and L1 major capsid protein of HPV respectively, in cervical squamous intraepithelial lesions with low and high grade, in order to determine, through morphological and clinical correlations, their applicability into practice when diagnosing and further monitoring the patients. There were 119 patients included in the study, having a mean age of 40, cytologically and histopathologically diagnosed in the Laboratory of Pathologic Anatomy of “Elena Doamna” Third Clinic of Obstetrics and Gynaecology in Iași. 42 of these patients were diagnosed with LSIL (low grade squamous intraepithelial lesion) and 51 with HSIL (high grade squamous intraepithelial lesion). The cervical-vaginal smears were interpreted using the Papanicolaou method. The conventional smears were assessed for the immunexpression of L1 capsid protein HPV, and the corresponding biopsies for p16 immunexpression. The colouring pattern of p16 protein was predominantly nuclear, with an occasional cytoplasmic positivity. P16 biomarker was positive in cytological smear prepared in a liquid environment for 71.6% of the patients, without significant differences for those over 40 years old (69.6% vs 69.0%;  $p=0.887$ ), with an increase in positivity from 54.8% in LSIL to 98% in HSIL ( $p=0.05$ ); the oncogenic risk was 1.55 higher ( $RR=1.55$ ;  $IC95\%: 1.19\div 2.01$ ). L1 protein was detected in 34.5% of the patients, the expression tending to increase in parallel with the increase in the severity of the lesions (66.7% LSIL; 17.6% HSIL). The presence of L1 protein in the patients with an increased risk of malignant transformation of HPV seems to be a protective factor ( $RR=0.42$ ;  $IC95\%: 0.27\div 0.66$ ). The immunexpression of L1 HPV protein has clinical applications in assessing the progression of cervical pre-cancer lesions. The analysis of p16 status, in parallel with the expression of L1 HPV protein, can be very useful in assessing the risk of progression for cervical intraepithelial squamous lesions. The preventive conduct supported by a primary care screening, leads to a decrease in the morbidity by pre-invasive lesions and an evolution with a favourable prognostic.

### INTRODUCTION

It is generally accepted nowadays that invasive cervical squamous tumours and the corresponding preceding lesions are caused by specific types of human papilloma virus (HPV), especially by the types with oncogenic risk that infect the anogenital tract. Now, the proportion of cervical carcinoma attributed to HPV infection is estimated at 99%. Many observations showed the importance of the immune answer in HPV infection. Previously there have been studies on the antibodies against capsid protein of different HPV types, using bacterial fusion proteins or chemically synthesized peptides (Achim, R., 1998; Alexandrescu, D., 1984). As an essential condition of these studies, we had to identify the type of infecting HPV. Until recently, the detection of HPV type in a certain tissue has been done through methods of hybridization with nucleic acids. The polymerase chain reaction (PCR) was introduced as a more effective and sensitive method of amplifying the DNA of HPV, being used both for general detection and for finding the type of HPV, particularly in genital infections. The problem of quantification is the main limit of technique, being difficult to distinguish between a latent infection (subclinical) and the obvious clinical lesions. As an alternative to hybridization and PCR, the immunological detection of viral capsid antigen can be used for diagnosing the productive HPV infections.

P16 tumour suppressor protein is a cyclin-dependent kinase inhibitor that regulates the transition from phase G1 to phase S in the cellular cycle (Altekruse, S.F., et al., 2003; Ancar, V., 1999).

The intense immunexpression of p16 was previously reported as being characteristic to the dysplastic and neoplastic cervical epithelium (Anderson, M. et al., 1992; Anderson, M. et al., 1996; Anderson, N.H., 2000). Overexpression of p16 slows down the cell cycle by inactivating the cyclin-dependent kinases that phosphorylate the retinoblastoma protein (pRb) (Andre, F.E., 2003; Anhang, R., et al., 2004). The viral oncogenes of HPV - E6 and E7, whose expression is associated with the malignant transformation of cervical epithelial cells (An, H.J., et al., 2003; Anghel, R. & Bălănescu, I., 1996), can tie to and inactivate pRb which, in turn, influences the expression of p16 protein in cervical intraepithelial squamous lesions (SIL) (Anton, G. & Socolov, D., 2000). Recent studies have concluded that p16 is a useful marker for the high risk HPV cervical neoplasia (9, 13) and also for assessing the progression of SIL (Arbyn, M., et al., 2010; Ardeleanu, C, et al., 1999). The behaviour of cervical intraepithelial squamous lesions is unpredictable, many of them, particularly the low grade ones being able to disappear without treatment. Invasive cervical carcinoma appears in about 10% of the intraepithelial lesions

preceding cancer, being strongly associated with HPV infection (Baccard-Longere, M., 1999; Badea, M. & Virtej, P., 2002; Baldauf, J.J., et al., 1997).

## PURPOSE AND OBJECTIVES

The purpose of this study was to accomplish a comparative assessment between the immunohistochemical and immunocytochemical expression of p16 protein and L1 capsid protein of HPV respectively, in the cervical intraepithelial squamous lesions of low and high grade, in order to determine, through morphological and clinical correlations, their practical applicability in diagnosing and further monitoring the patients.

## MATERIAL AND METHODS

There were 119 patients included in the study, all of them diagnosed cytologically and histopathologically in the Laboratory of Pathological Anatomy of “Elena Doamna” Third Clinic of Obstetrics and Gynecology in Iasi. 42 of these cases were diagnosed with LSIL (low grade squamous intraepithelial lesion) and 51 cases with HSIL (high grade squamous intraepithelial lesion), which needed further biopsy. The cervical-vaginal smears were fixed and coloured using the Papanicolaou method. The conventional smears were assessed for the immunoeexpression of L1HPV capsid protein, and the corresponding biopsies for the p16 immunoeexpression.

After establishing the cytodiagnostic, the cervical-vaginal smears were used for detecting the L1 HPV capsid protein through immunocytochemistry, using monoclonal antibodies (Cytoactiv HPV L1 High Risk Set REF SCA0850, Cytoimmun Diagnostics GmbH), following a standard protocol. Epithelial cells with a positive nuclear colouring received a positive score, considering that one coloured nucleus is enough for accomplishing the score.

Cervical biopsies were investigated through a histopathologic and immunohistochemical routine examination, using p16-D25 antibodies. The collected tissues were fixed for 24 hours in buffered formalin and were processed for inclusion in paraffin. Serial sections of 4-5 µm were removed the paraffin and were coloured with haematoxylin-eosin. After the standard histopathologic examination, we made further sections for the immunohistochemical examination. HIER (Heat-induced epitope retrieval) technique was used with a solution of Target Retrieval with pH 6 (cod S1700, DAKO, Denmark). After being blocked with endogene peroxidase and being non-specific linked, the sections were incubated with one of the primary antibodies, a monoclonal anti-p16 mouse (clone D25, cod sc-81613, Santa Cruz, USA) antibody, with a dilution of 1:100. The immune reaction was amplified using the corresponding secondary antibody and the Streptavidin–Biotin–Peroxidase HRP (cod K5001, DAKO, Denmark) complex. The sections were afterwards developed, using 3,3'-diaminobenzidine tetra hydrochloride (DAB) (cod K5001, DAKO, Denmark) chromogen, under microscopic control. The sections were finally counter-coloured with Mayer haematoxylin. There was also a negative control performed.

The quality control represented by external and internal negative and positive controls was necessary for monitoring the accuracy of tissue processing, colouring procedures and efficiency of reactives. The specificity of the primary antibody must be assessed through its negative controls. P16 protein was given a score, considering the estimating proportion of immunopositive cells (table 1).

**Table 1. p16 - immunohistochemical score**

Score	p16
0	absent
1	Weak (<25% immunopositivity)
2	Moderate (25-75% immunopositivity)
3	Intense (75-100% immunopositivity)

## RESULTS AND DISCUSSION

The positivity of **p16 biomarker** in cytological smear prepared in a liquid environment showed in 71.6% of the patients, with no significant differences for those over 40 years old (69.6% vs 69%; p=0.887).

In the cytological smears that were analysed, the distribution showed an increased p16 positivity from 54.8% in LSIL to 98% in HSIL (table 2.).

**Table 2. The detection rate of p16 biomarker in cytological smear in liquid environment**

Cytological diagnostic	Total number of cases	p16 positive biomarker	
		n	%
Negative	15	0	0.0
ASCUS	11	5	45.5
LSIL	42	23	54.8
HSIL	51	50	98.0
Total	119	78	65.5

79,6% of the patients with an increased risk of malignant transformation showed a positivity of the p16 biomarker, while the patients with a low risk of malignant transformation of HPV showed no case with a positive p16 ( $p < 0.001$ ). The association of an increased risk of HPV with p16 positivity induces a relative viral oncogenic risk 1,55 higher (RR=1.55; IC95%: 1.19÷2.01).

**L1 HPV capsid protein.** In the smear prepared in a liquid environment, L1 protein was detected in 34,5% of the patients. The distribution of the patients on age groups showed the presence of L1 in 31/73 patients under 40 years old (42.5%), compared with 10/36 patients over 40 years old (27.8%), but this distribution is not significant from the statistic point of view ( $p = 0.201$ ).

The study group showed an anti-L1 HPV immunoreactivity in 18.2% of the cases of ASCUS, 66.7% LSIL and 17.6% HSIL, this indicating that the expression of L1 protein tends to decrease in parallel with an increase in the severity of the lesions (table 3.).

**Table 3. The detection rate of L1 capsid protein in the cytological smear in liquid environment**

Cytological diagnostic	Total number of cases	positive L1 capsid protein	
		n	%
NEGATIVE	15	2	13.3
ASCUS	11	2	18.2
LSIL	42	28	66.7
HSIL	51	9	17.6
Total	119	41	34.5

29.1% of the patients with an increased risk of malignant transformation were detected with the presence of L1 capsid protein, which is significantly lower than the 100% patients with a low risk of malignant transformation of HPV ( $p = 0.00003$ ).

It was statistically proven that the presence of L1 protein in the patients with an increased risk of malignant transformation of HPV is a protective factor, because the relative risk is below 1 (RR=0.42; IC95%: 0.27÷0.66).

HPV infection was confirmed morphologically through the presence of the cytopathic effect of the virus (koilocytes) on smears and biopsies.

The colouring pattern of p16 protein was predominantly nuclear, with an occasional cytoplasmic positivity. Most cases showed heterogeneous colouring, with positive and negative cells.

From all cervical biopsies, p16 was positive in 54.8% in LSIL and 98% in HSIL. The ratio of biopsies with an intense immunorexpression of p16 increased in parallel with the severity of cytologic anomalies. In HSIL cases, the distribution of colouring was as follows: 65% of the whole epithelium (fig. 3), 35% of the base and intermediary layers. The intensity of colouring for HSIL cases was intense in 80% (fig. 1, 2), moderate in 16% and weak in 4%. In the case of LSIL category, the distribution of colouring was as follows: basal in 75% of the cases and occasional in 25%. There was no case with LSIL that showed a positive colouring of p16 all over the epithelium. The intensity of colouring in the cases diagnosed with LSIL was intense in 20% (fig. 3, 4, 5), moderate in 13% and weak in 67%.

From all cervical smears, L1 HPV capsid protein was shown in 66.7% of LSIL and 17.6% of HSIL. The expression of L1 capsid protein was significantly reduced for the cases diagnosed with HSIL, HPV positive. In the cases of LSIL, that were HPV positive we could not show a significant decrease in the expression of capsid L1 protein.

The positive reaction was characterized through an intense colouring of the whole nucleus, surrounded by cytoplasm without background colouring. In most cases, the positive reaction for HR-HPV L1 was positive in the typical koilocytes and in diskeratocytes, showing nuclear characteristics for HSIL (CIN 2 or CIN 3). In the cases of LSIL, the positivity of nuclei was present only in the koilocytes with characteristic morphology (fig. 6).

All the cases that were HPV positive (with morphological signs of HPV infection) were also p16-positive, without identifying any significant relation between the immunopositivity of HPV infection (active infection) and the intensity and distribution of p16.

L1 capsid protein is expressed in the active phase of HPV infection and is necessary in completing the viral cell cycle. Hence, the detection of the viral protein, through an immunohistochemical reaction, represents the proof of HPV infection in the examined tissues (Ball, C. & Madden, L.E., 2003). L1 viral capsid protein is considered a major target for the cell immune response (Baseman, J.G. & Kautsky, L.A., 2005). Moderate LSIL and SIL, without the immunohistochemical detection of L1 protein, are correlated in more than 80% of the cases, with progression of dysplasia. Griesser and colab. certify these aspects, underlining the fact that minor and moderate lesions without the expression of L1 capsid protein are significantly more exposed to progression, in comparison with the cases of positive L1 (Basen-Engquist, K., et al., 2003; Benagiano, G., et al., 2006). Most likely, the lack of HPV antigen is caused by a weak proteic synthesis, below the minimum level of immunohistochemical testing. Considering the fact that L1 represents the major target of the immune cellular response (Bibbo, M., et. al, 2002), a deficitary translation can lead to an inefficient depuration of the infected cells, promoting the integration of viral ADN in the genome of the host cell and transformation of the immature epithelial cells. The observation that a decreased positivity of HPV16 capsid protein in the serum of the patients who were diagnosed with cervical cancer is a reserved prognostic indicator, supports the importance of the specific umoral response. The immunocytochemical detection of L1 capsid, on conventional smears, can show the defence status that is locally induced on HPV infection and can offer prognostic information, especially for LSIL lesions.

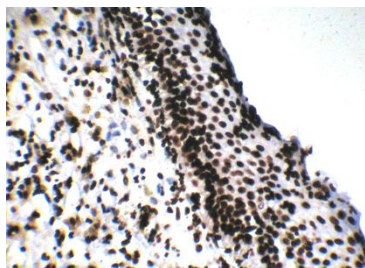


Fig. 1. HSIL (CIN2), p16, colouring intense immune colouring (x10) (x10)

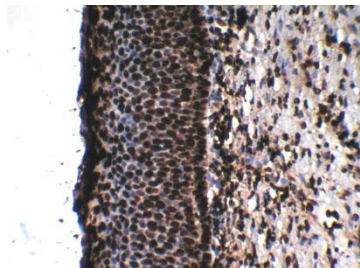


Fig. 2. HSIL (CIN3), p16, intense immune colouring for the entire thickness of the epithelium

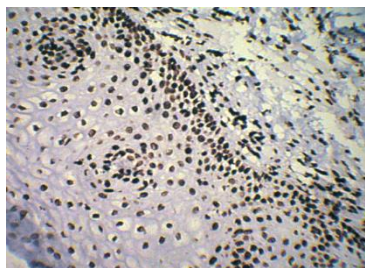


Fig. 3. LSIL, p16, intense immune colouring (x10) (x10)

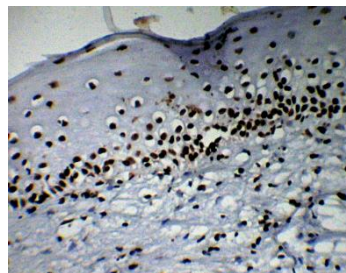


Fig. 4 LSIL, p16, intense immune colouring

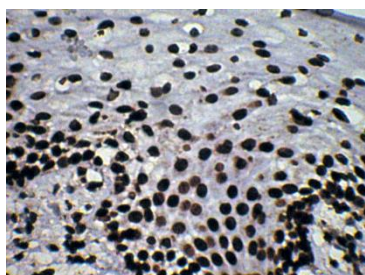


Fig. 5. LSIL, p16, intense immune colouring (x20)

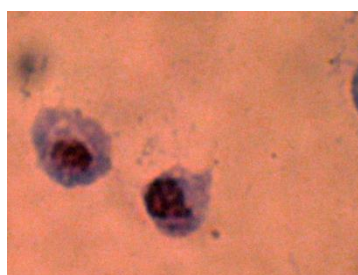


Fig. 6. LSIL, positive L1 HPV (x100)

## CONCLUSIONS

In our study, from all the cervical biopsies, p16 was positive in 54.8% of LSIL, 98% of CIN2 and of CIN3.

From all cervical smears, L1 HPV capsid protein was present in 68.8% of LSIL and 29.1% of HSIL.

The expression of L1 capsid protein was significantly reduced for the cases of positive HSIL, HPV. In the cases of positive LSIL, HPV, we could not demonstrate a significant decrease of the expression of L1 protein.

The analysis of p16 status, in parallel with the expression of L1 HPV protein, can be very useful in assessing the risk of progression for the cervical intraepithelial squamous lesions. A preventive conduct that is also supported by primary care screening, leads to a decrease of morbidity by preinvasive lesions and an evolution with a favourable prognostic.

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